

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 5

invention originally claimed for the following reasons: claims 107-109 are drawn to original Group IX, claims 37-39, drawn to a therapeutic agent comprising said antibody and cytotoxic agent, classified in class 424, subclass 138.1. The Examiner Stated that claims 110-112 are drawn to original Group X, claims 39-45, drawn to a method of detection using said antibody and composition comprising said antibody and carrier (or radioisotope), classified in class 435, subclass 7.1.

The Examiner stated that since applicant has received an action on the merits for the originally presented invention, this invention has been constructively elected by original presentation for prosecution on the merits. The Examiner stated that claims 107-112 are withdrawn from consideration as being directed to a non-elected invention and cited 37 CFR 1.142(b) and MPEP § 821.03.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 107-112 without prejudice to their right to pursue the subject matter of these claims in a later-filed application. Moreover, applicants respectfully request the examination of newly added claims 123-126. Newly added claim 123 recites as follows: A composition of matter comprising the antibody of any one of claims 114-121 and an agent conjugated to the antibody. Newly added claim 124 recites as follows: The composition of matter of claim 123, wherein the agent is a radioisotope or toxin. Newly added claim 125 recites as follows: A composition comprising a carrier and the composition of matter of claim 123. Newly added claim 126 recites as follows: A method of imaging prostate cancer in a subject which comprises administering to the subject the composition of matter of claim 123, wherein the agent is an imaging agent under conditions permitting formation of a complex between the composition of matter and prostate specific membrane

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 6

antigen, and obtaining an image of any complex so formed. Applicants contend that there is no additional burden on the Examiner to examine these claims since they all center around applicants' discovery of an antibody which binds to prostate specific membrane antigen polypeptide. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of objection and examine claims 114-126.

Rejection under 35 U.S.C. 102(b)

The Examiner rejected claims 98 and 105-106 under 35 U.S.C. 102(b) as anticipated by Feng et al 1991. The Examiner stated that Feng et al discloses of an isolated PSM antigen with a molecular weight of 100 kda and a monoclonal antibody which reacts with said antigen.

The Examiner stated that applicant's arguments filed 6/17/99 have been fully considered but they are not persuasive. The Examiner stated that applicant argues that the declarations of Kaladas, Rodwell, and Horoszewicz establish that the Feng reference is not enabling due to the lack of public availability of the 7E11-C5 hybridoma cell line. The Examiner stated that however, the 102(b) statute above clearly states that if the invention was described in a printed publication or in public use, the statute would apply. The Examiner stated that it is not required that the invention be described and be in public use. The Examiner stated that regarding Applicant's arguments that the disclosure of the specific 7E11-C5 antibody should not anticipate a genus claim, since the Patent Office does not have the facilities for examining and comparing applicant's proteins with the proteins of the prior art reference, the burden is upon applicants to show an unobvious distinction between the material structural and functional characteristics of the claimed proteins and the proteins of the prior art. The Examiner Stated that see In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and In re

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 7

Fitzgerald et al., 205 USPQ 594.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claims 98 and 105-106 without prejudice to their right to pursue the subject matter of this claim in a later-filed application and added new claims 114-126.

The Examiner has maintained his rejection of the claims under 35 USC §102(b). However, applicants respectfully submit that the public was not in possession of applicants claimed invention, i.e. an antibody which binds to prostate specific membrane antigenic polypeptide, before their effective filing date.

Declaration of Julius S. Horoszewicz Under 37 C.F.R. §1.132

The Declaration of Julius S. Horoszewicz ("Horoszewicz") establishes the following:

- 1) Julius S. Horoszewicz did not make publicly available the 7E11-C5 hybridoma cell line or the 7E11-C5 monoclonal antibody prior to November 10, 1992.
- 2) The only persons who had either the 7E11-C5 hybridoma cell line or the 7E11-C5 monoclonal antibody were (a) employees or agents of Cytogen, or (b) persons who received them under an agreement with Cytogen restricting their use.

Declaration of John D. Rodwell, Ph.D. Under 37 C.F.R. §1.132

The Declaration of John D. Rodwell ("Rodwell") establishes the following:

- 1) Cytogen received the 7E11-C5 hybridoma cell line after signing an exclusive license agreement with Julius S. Horoszewicz on April 20, 1989 with respect to the 7E11-C5 hybridoma cell line or the 7E11-C5 monoclonal antibody.
- 2) Cytogen did not distribute the 7E11-C5 hybridoma cell line to any person or entity prior to November 10, 1992.

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 8

3) CytoGen did not distribute the 7E11-C5 monoclonal antibody to any person or entity other than Eastern Virginia Medical School prior to November 10, 1992. Eastern Virginia Medical School received the antibody pursuant to a restrictive agreement.

Second Declaration of Paul Kaladas, Ph.D. Under 37 C.F.R. §1.132  
The Second Declaration of Paul Kaladas ("Kaladas 2"), attached hereto as Exhibit A, establishes the following:

- 1) Neither the 7E11-C5 prostate carcinoma associated antigen nor a nucleic acid encoding it had been isolated or characterized prior to the publication of the Feng et al. abstract.
- 2) Feng et al does not enable one skilled in the art to make the 7E11-C5 antibody per se.
- 3) In order to make an antibody having the properties of the 7E11-C5 antibody, one skilled in the art would need purified 7E11-C5 prostate carcinoma associated antigen.
- 4) To obtain purified 7E11-C5 prostate carcinoma associated antigen, one skilled in the art would need to have either:
  - a) an antibody such as the 7E11-C5 antibody;
  - b) a hybridoma cell line such as the one which produces the 7E11-C5 antibody; or
  - c) a nucleic acid encoding the 7E11-C5 prostate carcinoma associated antigen.
- 4) The following were not available prior to November 5, 1992:
  - a) the hybridoma cell line which produced the 7E11-C5 antibody;
  - b) the 7E11-C5 antibody per se;
  - c) a similar antibody which recognized the prostate carcinoma associated antigen; and
  - d) a nucleic acid encoding the prostate carcinoma associated antigen.
- 5) Feng et al. does not describe properties of the 7E11-C5 prostate carcinoma antigen or any procedure that would have

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 9

enabled one skilled in the art to obtain purified 7E11-C5 prostate carcinoma antigen without the use of a specific antibody such as the 7E11-C5 antibody.

- 6) Feng et al. did not enable one skilled in the art to obtain purified 7E11-C5 prostate carcinoma antigen.
- 7) Feng et al. did not enable one skilled in the art to make the 7E11-C5 antibody per se or a similar antibody.

The Examiner has rejected the claims as anticipated by Feng et al. It appears that the Examiner is alleging that Feng et al describes the 7E11-C5 antigen and antibody such that this reference anticipates the claimed invention. Applicants respectfully submit that Feng et al does not anticipate the claimed invention.

Neither the 7E11-C5 antibody nor the hybridoma cell line which produces this antibody were in public use

Applicants respectfully submit that neither the 7E11-C5 antibody nor the hybridoma cell line which produces this antibody were in public use. The Federal Circuit has clearly established that "public use" includes a use of the invention by some person other than the inventor who is under **no limitation, restriction, or obligation of secrecy** to the inventor. See Petrolite Corporation v. Baker Hughes Incorporated, 96 F.3d 1423 (Fed Cir 1996). Emphasis added.

Horoszewicz establishes that the only persons who had the 7E11-C5 antibody or hybridoma cell line were employees or agents of Cytogen, or those who received them under an agreement from Cytogen restricting their use.

Rodwell establishes that Cytogen received the hybridoma pursuant to an exclusive license agreement, and that the only entity to whom they distributed the antibody was Eastern Virginia Medical School who received it pursuant to a restrictive agreement.

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 10

Accordingly, prior to applicants' effective filing date, the only persons or entities who had the antibody and/or hybridoma were (1) Horosewicz, the inventor; (2) Cytogen, who received it pursuant to an exclusive license agreement; and (3) Eastern Virginia Medical School, who signed a restrictive agreement. Accordingly, applicants contend that neither the 7E11-C5 antibody nor the hybridoma cell line were publicly available prior to their effective filing date, i.e. November 5, 1992.

Feng et al is not enabling to make the antibody

Applicants respectfully submit that Feng et al does not anticipate applicants' claimed invention. The standard in determining whether an invention is anticipated by a reference is whether the reference contains an enabling disclosure. See In re Hoeksema, 399 F.2d 269 (CCPA 1968).

Kaladas 2 establishes that the description in Feng et al would not have enabled one skilled in the art to make the 7E11-C5 antibody per se. Kaladas 2 further establishes that Feng et al would not have enabled one skilled in the art to make an antibody having the properties of the 7E11-C5 antibody because one would need purified 7E11-C5 prostate carcinoma associated antigen. However, to obtain purified 7E11-C5 prostate carcinoma associated antigen, one would need one of the following:

- 1) an antibody such as the 7E11-C5 antibody. However, this antibody was not available to the public. See the Kaladas 2, Horosewicz and Rodwell declarations, and *supra* pages 9-10;
- 2) a hybridoma cell line such as the one which produces the 7E11-C5 antibody. However, the 7E11-C5 hybridoma cell line was not publicly available. See the Kaladas 2, Horosewicz and Rodwell declarations, and *supra* pages 9-10; or

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 11

3) a nucleic acid encoding the 7E11-C5 prostate carcinoma associated antigen. However, such nucleic acid was not publicly available. See Kaladas 2 declaration.

Kaladas 2 further establishes that Feng et al does not describe properties of the antigen or any procedure that would have enabled one skilled in the art to obtain purified 7E11-C5 prostate carcinoma associated antigen without the use of a specific antibody such as the 7E11-C5 antibody. However, neither the 7E11-C5 antibody per se nor a similar antibody were publicly available. See Kaladas 2, Horosewicz and Rodwell declarations. Accordingly, Kaladas 2 establishes that Feng et al would not have enabled one skilled in to obtain purified 7E11-C5 prostate carcinoma antigen. And therefore, Feng et al would not have enabled one skilled in the art to make the 7E11-C5 antibody per se or a similar antibody. Accordingly, Feng et al is not enabling to make the antibody and therefore, does not anticipate applicants' claimed invention and should be removed as a reference.

Applicants contend that the above remarks establishes that Feng et al does not anticipate applicants claimed invention and therefore should be removed as a reference. Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

**Rejection under 35 U.S.C. 112, first paragraph**

The Examiner rejected claim 113 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner stated that the specification as originally filed provides no support for the concept of a genus of antibody which

Applicants: Ron S. Israeli, et al.  
Serial No.: 08/705,477  
Filed : August 29, 1996  
Page 12

binds to prostate specific membrane antigen other than the known 7E11-C5 monoclonal antibody. The Examiner stated that any negative limitation or exclusionary proviso must have basis in the original disclosure, and cited *Ex parte Graselli*, 231 USPQ 393 (Bd. Pat. App. 1983) aff'd mem., 738F. 2d 453 (Fed Cir. 1984).

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claim 113 without prejudice to their right to pursue the subject matter of this claim in a later-filed application. Applicants contend that this amendment obviates the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Supplemental Information Disclosure Statement

In accordance with their duty of disclosure under 37 C.F.R. §1.56 applicants would like to direct the Examiner's attention to the following references which are listed on the attached Form PTO-1449 (**Exhibit B**) and attached hereto as **Exhibits 1-2**:

1. Kay et al., U.S. Patent 5,852,167, issued December 22, 1998 (**Exhibit 1**).
  
2. Wright et al., U.S. Patent 5,153,118, issued October 6, 1992 (**Exhibit 2**); and

Summary

In view of the foregoing remarks, applicants respectfully request that the above grounds of rejection and objection be reconsidered and withdrawn and earnestly solicit allowance of the now pending claims.

If a telephone interview would be of assistance in advancing

Applicants: Ron S. Israeli et al.  
Serial No.: 08/470,735  
Filed : June 6, 1995  
Page 13

prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee, other then the enclosed \$604.00 fee which includes the \$435.00 fee for a three month extension of time and the \$169.00 for additional claims, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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Reg. No. 28,678

Original Articles

# Expression of Prostate-Specific Membrane Antigen in Normal, Benign, and Malignant Prostate Tissues

George L. Wright, Jr, PhD,\*†‡§ Cara Haley, BS,\*‡§ Mary Lou Beckett, MS,\*‡§ and Paul F. Schellhammer, MD\*†‡§

Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein recognized by the murine monoclonal antibody (MAb) 7E11-CS.3 both in its native (CYT-351) and immunoconjugate form (CYT-356). Previous studies have shown that tissue expression of PSMA is highly restricted to prostate tissues. In this study, a definitive immunohistochemistry evaluation was performed to assess PSMA expression in prostate tissues. A stain index was established by multiplying the percentage of stained cells by the intensity of the stained cells to provide a quantitative measurement of PSMA expression in the various tissue types. The cellular location of PSMA, its correlation with clinical status, and its comparison with the expression of prostate-specific antigen (PSA) were evaluated. Prostate-specific membrane antigen was found to be highly expressed in most of the normal intraepithelial neoplasia, and the primary and metastatic prostate tumor specimens evaluated. In contrast to PSA, PSMA expression was often heterogeneous with variable staining patterns, ranging from a low-level diffuse cytoplasmic staining in normal prostate epithelium to very intense cytoplasmic and focal membrane staining in high-grade primary carcinomas and metastatic tissues. The predominant cytoplasmic staining was expected because the antigenic epitope of the PSMA transmembrane glycoprotein recognized by MAb 7E11-CS.3 is located in the cytoplasmic domain. Benign prostate tumors, ie, hypertrophy, showed the lowest expression of PSMA with a stain index of 52, compared with stain indexes of 146 and 258 for normal prostate and bone metastatic tissues, respectively. The reason for the apparent down-regulation of PSMA in benign prostate tissue is unknown but may be related to a splicing variant or post-translational modification of PSMA. Expression of PSMA was observed to increase with increasing pathologic grade, but not with clinical stage. Although PSMA was overexpressed in poorly differentiated and metastatic prostate tumors, expression in the primary tumor did not correlate with nodal status, extracapsular penetration, or seminal vesicle invasion. These results suggest that PSMA is not a

useful biomarker of disease progression; however, high expression does appear to be associated with the more aggressive prostate carcinoma phenotype. The restricted specificity, differential prostate tissue expression, and overexpression of PSMA in metastatic tissues support the continued study of this unique prostate tumor-associated biomarker for developing new strategies for diagnosis and therapy of prostate cancer. (*Urol Oncol* 1995;1:18-28)

**P**rostate cancer is the most common (noncutaneous) cancer diagnosed in the American male and is steadily increasing, not only as a result of an increasing population of older men, but also because of greater awareness of the disease and earlier diagnosis using tumor markers such as prostate-specific antigen (PSA). It is projected that 200,000 men were diagnosed with prostate cancer in 1994,<sup>1</sup> representing a 34% increase in the number of prostate cancer cases (165,000) diagnosed in 1993. If the 1994 estimate is accurate, prostate cancer will become the most commonly diagnosed cancer, exceeding breast cancer (183,000) by 27,000 cases. More than 38,000 men are expected to die of prostate cancer in 1994, making deaths from prostate cancer second only to lung cancer deaths. Patients diagnosed with localized disease have far better survival rates than patients diagnosed with metastatic disease. Early detection of localized prostate cancer and improved treatment of metastatic disease are important strategies to reduce prostate cancer deaths.

Although serum PSA measurements have had a major impact on the diagnosis and management of prostate cancer,<sup>2,3</sup> PSA is far from being the ideal cancer marker. Twenty-five percent of patients with benign prostatic hypertrophy (BPH) present with elevated levels of PSA, approximately 30% of prostate cancer patients present with normal PSA values, and PSA expression is unable to differentiate biologically active from inactive cancers. These statistics suggest that other clinical markers are needed to improve early diagnosis, to identify aggressive tumors, and to develop new therapeutic strategies. A new prostate marker, prostate-specific membrane antigen (PSMA), may meet one or more of these objectives. Prostate-specific membrane antigen appears to be a transmembrane glycoprotein with a major Mr 100,000 component<sup>4-6</sup> recognized by monoclonal antibody (MAb) 7E11-CS.3.<sup>7</sup> Recently, the cDNA encoding PSMA was cloned, and the deduced amino acid sequence revealed a novel polypeptide

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structure.<sup>1</sup> Clinical trials using MAb 7E11-C5.3 conjugated to either <sup>111</sup>In or <sup>90</sup>Y are in progress for diagnostic imaging and therapy, respectively.<sup>8-11</sup> Previous reports<sup>5,7-9</sup> have shown that PSMA expression is highly restricted to prostate tissues, and that the expression in normal prostate tissues appeared to be less than that in malignant prostate tissues. These studies, performed primarily to determine the specificity of PSMA expression, evaluated a small number of tissue specimens, and no relation of PSMA expression to clinical status was presented. In this report, we present a definitive description of the differential expression of PSMA in normal prostate, BPH, prostate intraepithelial neoplasia (PIN), and primary and metastatic prostate carcinoma (CaP) tissues, and the relation of PSMA expression to tumor grade and extraprostatic disease.

## Materials and Methods

### Tissues

Formalin-fixed, paraffin-embedded blocks of transurethral resected specimens of BPH, prostatectomy specimens of prostate carcinoma, and lymph node and bone metastatic tissues were obtained from the Virginia Prostate Center Tissue Bank. Normal prostate tissue was obtained from males aged 16 to 45 years with no evidence of prostate disease. These tissues were obtained by autopsy, usually within 12 hours after death, from the Cooperative Human Tissue Network, University of Alabama at Birmingham; and from the Norfolk Medical Examiner's Office. All tissue specimens for paraffin embedding were fixed in the same neutral buffered 10% formalin. Samples of normal, BPH, and prostate carcinoma tissues obtained directly from surgery or autopsy also were embedded in OCT compound in cryomolds and snap-frozen in isopentane over liquid nitrogen.

### Monoclonal Antibodies

Affinity-purified MAb 7E11-C5.3 (referred to as native antibody or CYT-351) and an affinity-purified conjugated form of MAb 7E11-C5.3 (designated CYT-356<sup>12</sup>) were provided by CYTOGEN Corp. (Princeton, NJ). Monoclonal antibody EVMS-PSA-5 was produced in mice against purified PSA from pooled normal seminal plasma and affinity purified from ascites, following our published protocols.<sup>13-15</sup>

### Immunoperoxidase Staining

The expression of PSMA and PSA in tissues was detected by the avidin-biotin peroxidase assay using the ABC Elite Vectastain kit (Vector Laboratories, Burlingame, CA), as described previously.<sup>13-15</sup> Briefly, 4- $\mu$ m paraffin sections were cut, deparaffinized, and rehydrated through xylene and a graded series of alcohols. Endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes. Frozen sections were cut at 6  $\mu$ m, briefly dipped in cold acetone, and stored at -20°C until used, or air dried for 30 minutes before proceeding with the staining reaction. Endogenous peroxidase activity was blocked with 3.0% H<sub>2</sub>O<sub>2</sub> in water for 5 minutes.

From this point on, both frozen and paraffin-embedded sections were treated in the same manner. Nonspecific binding was blocked by incubation with 10% normal horse serum for 10 minutes, followed by a 30-minute incubation with the primary antibody, either MAb 7E11-C5.3 or PSA-5, followed by a 10-minute incubation with the biotinylated secondary antibody, and then the ABC complex. The optimal concentrations for the antibodies (20  $\mu$ g/mL for MAb 7E11-C5.3 and 2  $\mu$ g/mL for PSA-5) were predetermined by titrating the MAbs on normal, BPH, and prostate carcinoma tissues. These concentrations from the same antibody lot were used for evaluating all the specimens in this study. After development with the chromogen substrate 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO), the sections were counterstained with Mayer's hematoxylin and mounted in aqua mount (Lerner Laboratories, Pittsburgh, PA). Antigen expression was scored by calculating the percentage of cells positive in a number of 20-mm objective views sufficient to cover all areas of the tissue section. Staining intensity was also recorded using a scale of 1 (low), 2 (moderate), and 3 (high). The stained tissue sections were scored independently by two investigators, with the two scores having a difference of less than 10%. A stain index was calculated by multiplying the mean percentage PSMA-positive cells by the mean staining intensity.

## Results

### Expression of PSMA and PSA in Normal, Benign, and Malignant Prostate Tissues

The binding of MAb 7E11-C5.3 was compared with the binding of a MAb to PSA using the immunoperoxidase assay on frozen and formalin-fixed, paraffin-embedded tissue sections of prostate specimens consisting of normal prostate, BPH, and malignant primary and metastatic prostate carcinomas. By optimizing the staining conditions, both frozen (data not shown) and paraffinized tissue specimens from the same patient gave identical staining patterns for MAb PSA-5 and the native and immunoconjugate forms of MAb 7E11-C5.3. The use of paraffin-embedded tissues enabled us to conduct a large retrospective study to evaluate MAb 7E11-C5.3 reactivity on normal, benign, PIN, and malignant prostate tissues. The epithelial cells of all four prostate tissue types were found to express both the PSMA and PSA antigens (Table 1; Figures 1-4). As expected, nearly all the prostate specimens expressed PSA, with the exception of the bone marrow metastatic specimens, of which only 57% stained positive for PSA. The mean percentage of epithelial cells expressing PSA ranged from 80-98% for all prostate tissue types, with the exception of the bone metastatic specimens (48%). Ninety-one to 100% of the prostate tissues expressed PSMA, with the exception of the BPH specimens, in which PSMA expression was positive in only 22 of 27 (81%). The mean percentage of epithelial cells expressing PSMA in the PIN specimens was 59%, and in the primary prostate carcinomas was 53%. The highest expression was found in the metastatic tumors: 72% for lymph nodes and 92% for bone metastasis. Of special interest was the observation that the highest percentage

TABLE I. COMPARISON OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN WITH PROSTATE-SPECIFIC ANTIGEN EXPRESSION IN FORMALIN-FIXED, PARAFFIN-EMBEDDED PROSTATE TISSUES

Tissue	PSMA		PSA	
	No. positive/ no. tested	Mean % positive cells	No. positive/ no. tested	Mean % positive cells
Normal	12/12 (100%)	77	12/12 (100%)	98
BPH	22/27 (81%)	29	27/27 (100%)	95
PIN	21/21 (100%)	59	21/21 (100%)	98
CaP	157/165 (95%)	53	161/165 (98%)	81
LN mets	72/79 (91%)	72	74/79 (94%)	81
Bone mets	7/7 (100%)	92	4/7 (57%)	48

PSMA = prostate-specific membrane antigen; PSA = prostate-specific antigen; BPH = benign prostate hyperplasia; PIN = prostate intraepithelial neoplasia; CaP = primary prostate carcinoma; LN = lymph node; Mets = metastasis.

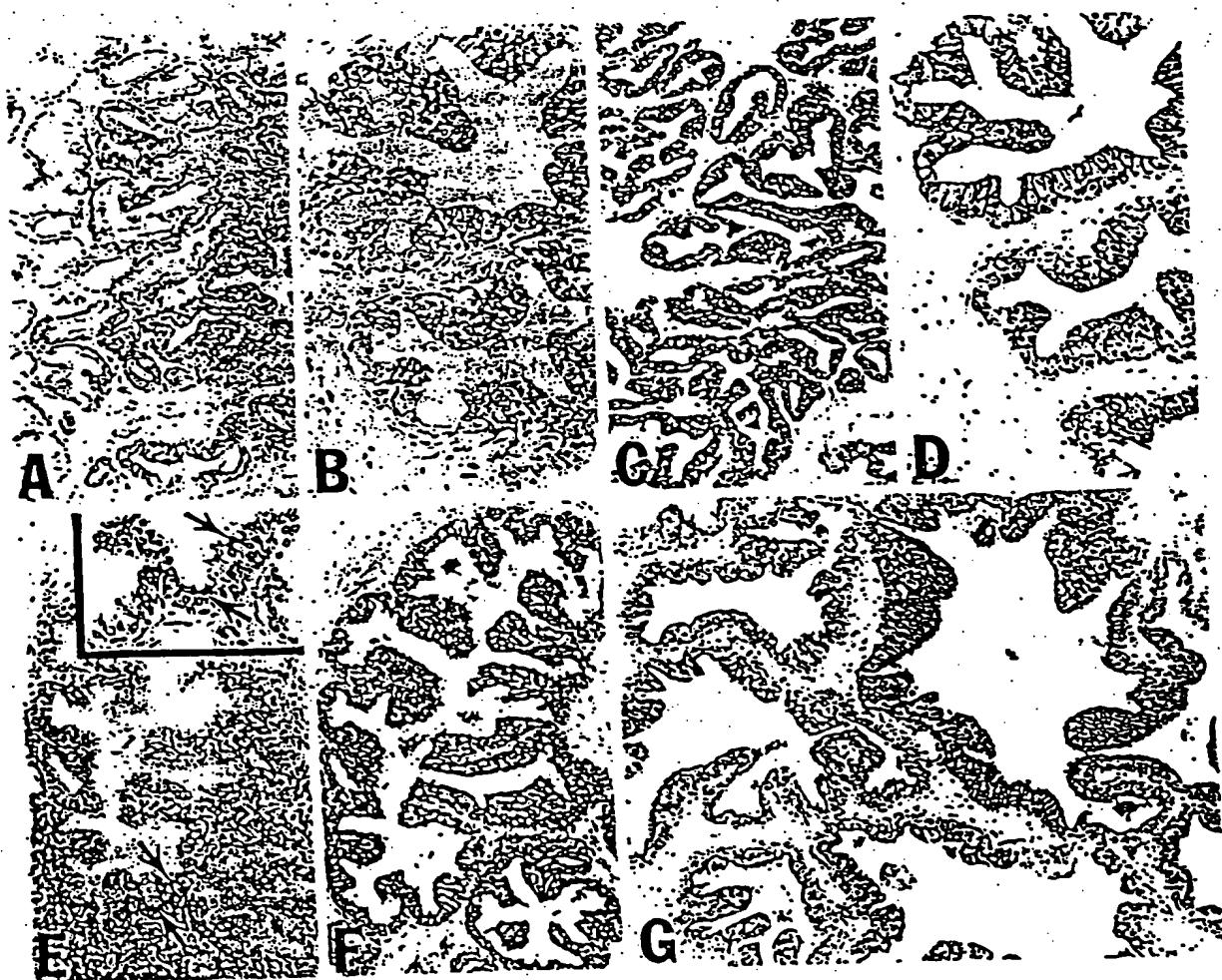
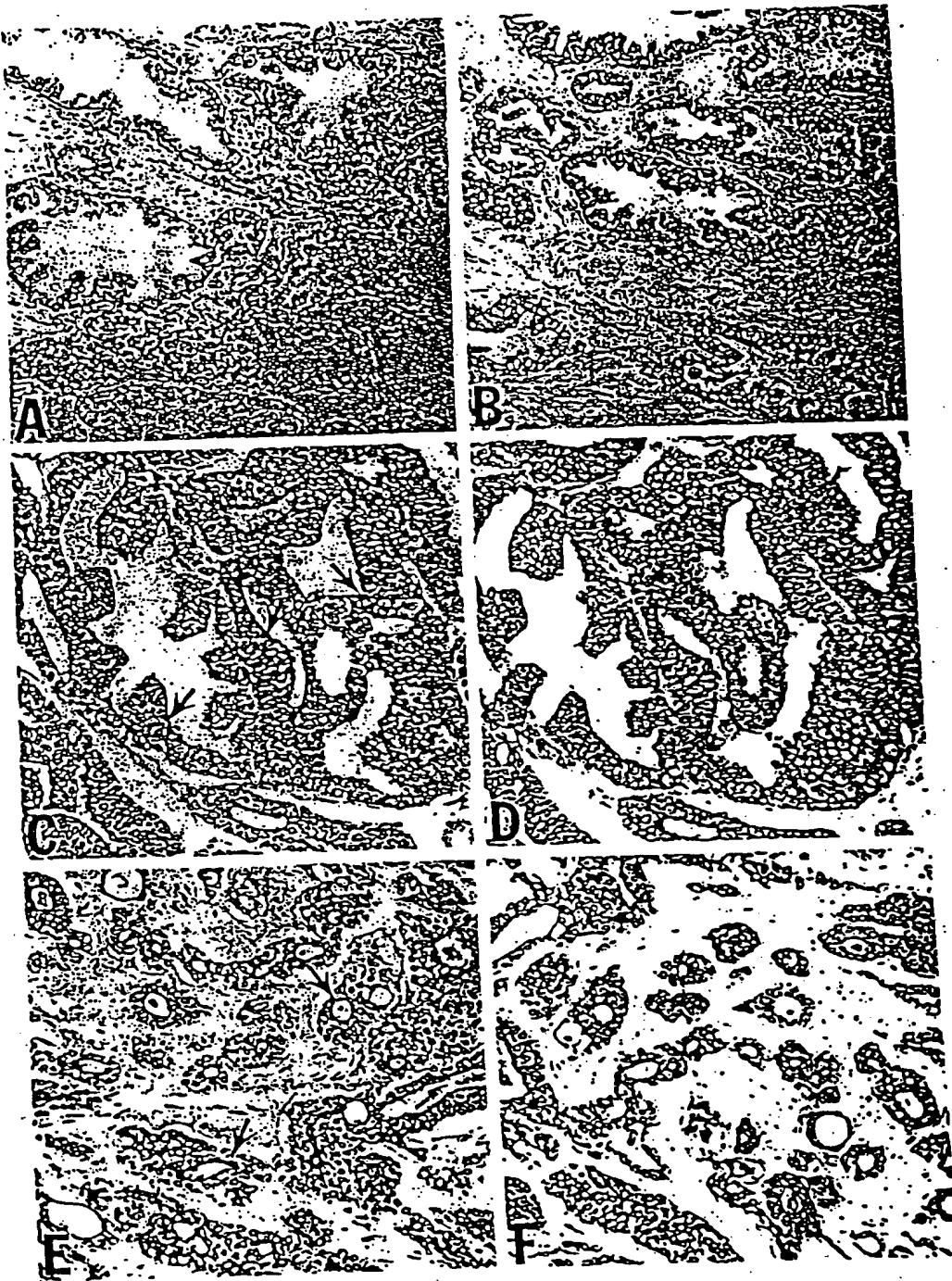
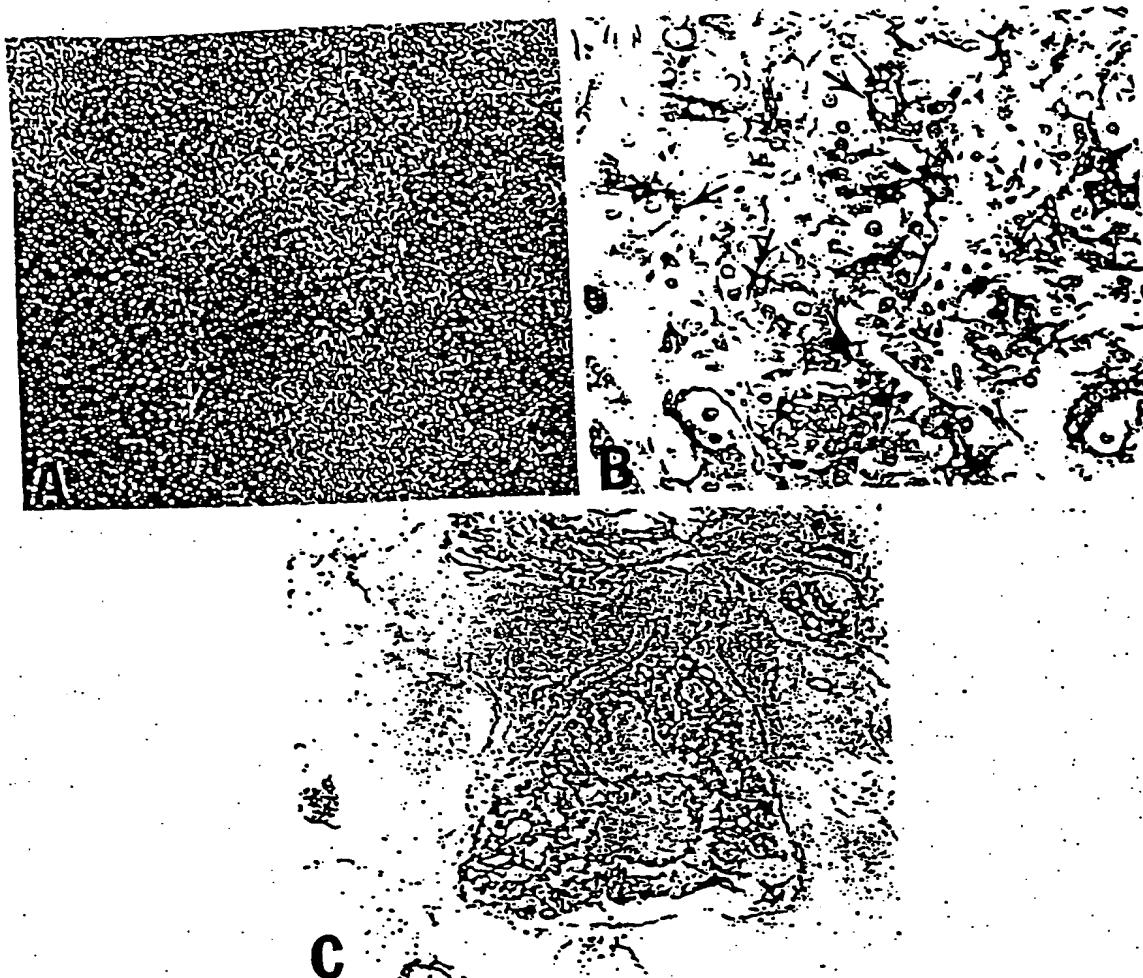


FIGURE 1. Immunoperoxidase staining of normal, benign (BPH), and PIN tissues with monoclonal antibodies to either PSMA or PSA. A-D) Normal prostate tissues. A) and B) stained for PSMA; C) and D) stained for PSA. Note diffuse cytoplasmic and low staining intensity for PSMA. E) and F) BPH tissue stained for PSMA (E) and PSA (F). Note scattered focal membrane staining for PSMA (arrows). G) PIN stained for PSMA. Original magnification:  $\times 100$  (A-C);  $\times 200$  (E-F);  $\times 400$  (E inset).



**FIGURE 2.** Prostate carcinoma tissues stained with monoclonal antibody to either PSMA or PSA. A) and B) Well-differentiated carcinoma; C) and D) moderately differentiated carcinoma; E) and F) poorly differentiated carcinoma. A), C), and E) stained for PSMA; B), D), and F) stained for PSA. Note the minimal and low-intensity cytoplasmic staining for PSMA in the well-differentiated carcinoma, with increase in the number of cells and staining intensity with increasing tumor grade, and intense focal and luminal membrane staining (arrows). Original magnification:  $\times 200$  (A-F).



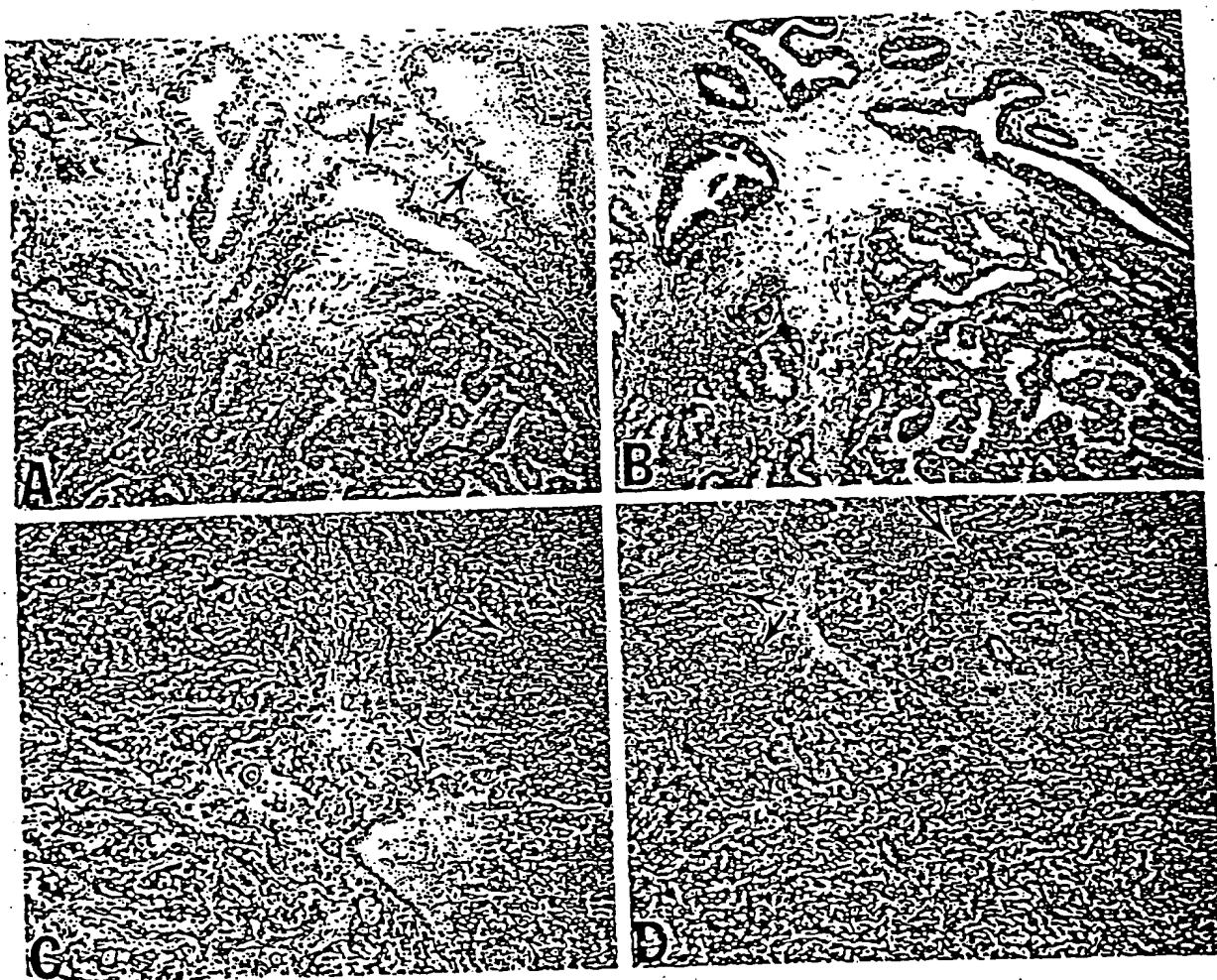
**FIGURE 3.** Metastatic prostate tissues stained for PSMA. A) and B) Lymph node metastatic tissue. C) Bone metastatic tissue. B) is a higher magnification showing intense focal and luminal membrane staining (arrows) associated with minimal cytoplasmic staining. Original magnification:  $\times 100$  (A,C);  $\times 400$  (B).

(77%) of epithelial cells expressing PSMA was in the normal prostate specimens, and the lowest percentage of positive cells (29%) occurred in the BPH specimens.

#### **Expression and Cellular Localization of PSMA in Prostate Tissues**

We evaluated PSMA expression in prostate tissues further by taking into consideration both the percentage and the staining intensity of prostate epithelial cells. A stain index was calculated by multiplying the mean percentage of cells expressing PSMA by the staining intensity (1 = low; 2 = moderate; 3 = high intensity). With this approach, BPH specimens clearly had the lowest stain index ( $SI = 52$ ), ie, the lowest number of positive epithelial cells and the lowest staining intensity (Table 2, Figure 1E). Both the number of stained cells and the staining intensity increased in the PIN (Figure 1G) and the malignant prostate specimens (Figure 2), with the highest index ( $SI = 258$ ) determined for the bone metastatic

specimens. Although the normal prostate tissues had a high stain index ( $SI = 146$ ), the staining pattern was quite different from that of the other tissue specimens. In these specimens, PSMA expression was predominantly diffuse and cytoplasmic with low to moderate staining intensity (Table 2; Figure 1B), with an occasional duct or luminal cell showing luminal membrane staining. Of all prostate tissues examined, BPH demonstrated the greatest antigenic heterogeneity. In contrast to normal prostate, few luminal cells expressed PSMA and the expression was often focal, with some apical membrane staining and minimal cytoplasmic staining (Figure 1E). The PIN lesions had a stain index ( $SI = 130$ ) similar to normal prostate but showed a more intense, diffuse cytoplasmic and luminal membrane staining (Figure 1G). The stain index ( $SI = 133$ ) for PSMA expression in the 165 primary prostate carcinomas evaluated, including all histopathologic grades, was similar to the index for normal and PIN tissues (Table 2). Cellular localization of PSMA in these tissues ranged from diffuse cytoplasmic staining in the well-differentiated speci-



**FIGURE 4.** Immunoperoxidase staining of three representative prostate carcinomas to illustrate the differential PSMA expression in various tissue types in the same specimen. A) and B) are serial sections from one patient; C) and D) are sections from two different patients, respectively. A), C), and D) were stained for PSMA and B) was stained for PSA. Note minimal to no staining in the normal/benign and well-differentiated (arrows) areas, compared with the intense focal and luminal membrane staining with some cytoplasmic staining (particularly in section D) in the poorly differentiated carcinoma areas. Note that all tissue areas stained for PSA (B). Original magnification:  $\times 100$  (A-D).

**TABLE 2. DIFFERENTIAL EXPRESSION OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN IN FORMALIN-FIXED, PARAFFIN-EMBEDDED PROSTATE TISSUES**

Tissue	No. positive/ no. tested	% positive cells <sup>a</sup>	Intensity <sup>a</sup>	Stain index <sup>b</sup>	PSMA localization <sup>c</sup>			
					DC	FC	F/M	LE
Normal	12/12 (100%)	77 $\pm$ 32	1.9 $\pm$ 0.33	146	++			+
BPH	22/27 (81%)	29 $\pm$ 29	1.8 $\pm$ 0.90	52	+		++	+
PIN	21/21 (100%)	59 $\pm$ 21	2.2 $\pm$ 0.37	130	+		++	
CaP	157/165 (95%)	53 $\pm$ 32	2.5 $\pm$ 0.66	133	++		++	+++
LN mets	60/64 (94%)	72 $\pm$ 36	2.7 $\pm$ 0.92	194	+	++	++	+++
Bone mets	7/7 (100%)	92 $\pm$ 10	2.8 $\pm$ 0.40	258	+	++	++	+++

<sup>a</sup> DC = diffuse cytoplasmic; FC = focal areas within the cytoplasm; F/M = membrane or focal membrane; LE = edge of luminal cells; other abbreviations as in Table 1.

<sup>b</sup> Mean  $\pm$  SD.

<sup>c</sup> Stain index calculated by multiplying the mean percentage of cells staining by the staining intensity.

<sup>a</sup> + = low staining; ++ = moderate staining; +++ = high staining.

TABLE 3. PROSTATE-SPECIFIC MEMBRANE ANTIGEN EXPRESSION VERSUS GRADE

Gleason sum	No. positive/ no. tested	Mean % cells positive	Mean stain intensity	Stain index
2-4 (WD)	22/26 (85%)	48	2.3	110
5-7 (MD)	92/100 (92%)	50	2.5	125
8-10 (PD)	37/39 (95%)	62	2.6	161

WD = well-differentiated; MD = moderately differentiated; PD = poorly differentiated.

mens (Figure 2A) to intense focal and luminal membrane staining in the moderate (Figure 2C) to high-grade carcinomas (Figure 2E). Perinuclear staining was sometimes observed in a few malignant epithelial cells in some of the high-grade specimens. Staining heterogeneity was evident for all tumor grades, with the highest degree observed in the low to moderate grades. The highest PSMA expression, in terms of number of cells staining and intensity of staining, was observed in the metastatic tissues (Table 2; Figure 3). As shown in Figure 3, the predominant cellular expression of PSMA in the lymph node and bone metastatic specimens was intense apical membrane staining, often with minimal cytoplasmic staining (Figure 3). Less common was the finding of occasional perinuclear staining and intensely stained focal areas

within the cytoplasm (not shown). The PSMA staining pattern observed in the metastatic tissues was similar to that for the moderate to high-grade prostate carcinoma specimens (Figure 2C and E); however, there was considerable less staining heterogeneity in the metastatic tissues.

#### PSMA Expression by Tumor Grade

Table 3 shows the expression of PSMA in the different pathologic grades. The Gleason scores of all 165 primary prostate carcinomas were placed into three groups representing the three general pathologic differentiation grades. Although not remarkable, a slight but positive correlation of PSMA expression with tumor grade was observed. The differential expres-

TABLE 4. COMPARISON OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN EXPRESSION IN LOW-GRADE AND HIGH-GRADE TUMOR AREAS ON THE SAME PROSTATE CARCINOMA TISSUE SPECIMEN

Tissue specimen	Low grade			High grade		
	%Cell <sup>a</sup>	In <sup>b</sup>	Index <sup>c</sup>	%Cell <sup>a</sup>	In <sup>b</sup>	Index <sup>c</sup>
CA153	20	1	20	95	3	285
CA1061	20	1	20	98	2.5	245
CA8750	10	1	10	70	3	210
CA5262	20	1	20	75	3	225
CA1124	70	3	210	90	3	270
CA1197	35	1	35	100	3	300
CA1789	50	1	50	50	2	100
CA8331	20	1	20	98	3	294
CA1022	5	1	5	90	2.5	225
CA2048	55	3	165	100	2	200
CA7506	70	2	140	95	3	285
CA4319	5	1	5	90	2	180
CA4475	35	2	70	90	3	270
CA3651	10	2	20	80	3	240
CA3984	20	1	20	60	3	180
CA8725	50	3	150	90	3	270
CA1471	85	2	170	100	3	300
CA6166	10	1	10	95	3	285
CA0600	40	2	80	70	3	210
CA5850	100	1	100	100	3	300
CA1977	65	3	195	80	3	240
CA1709	10	1	10	99	2.5	248

<sup>a</sup>%Cell = percentage of tumor cells staining.

<sup>b</sup>In = staining intensity (1 = low; 2 = moderate; 3 = strong).

<sup>c</sup>Staining index, calculated by multiplying the mean percentage of positive cells by staining intensity.

sion of PSMA in low- and high-grade carcinomas was more accurately accessed when separate indexes were calculated for the low- and high-grade areas contained in the same tumor specimen. Table 4 shows the results of PSMA expression in 22 randomly selected high-grade carcinomas containing focal areas of low-grade carcinoma. In all 22 cases, the stain index for the high-grade areas (mean SI = 244) was higher than that for the low-grade areas (mean SI = 59). Figure 4 shows the differential staining patterns in three carcinoma specimens containing both high- and low-grade tumor areas. Strong staining was observed in the poorly differentiated areas, often with minimal to no staining of the low-grade areas and the normal/benign areas. No correlation was found between PSMA expression and clinical or pathologic stage (data not shown).

### PSMA Expression in Lymph Node Metastases

The high PSMA expression in metastatic lymph nodes (94% of 64 positive nodes, SI = 194; Table 2) suggested that PSMA

expression in primary carcinomas may represent a biomarker of metastatic progression. Although the percentage of tumor cells expressing PSMA often was increased in the metastatic lymph node (Table 5), the patient's primary carcinoma did not reflect this PSMA activity; therefore, PSMA staining in the primary tumor was not predictive of nodal status (Table 6). Similarly, PSMA expression did not correlate with positive margins, extracapsular penetration, or seminal vesicle invasion (data not shown). Correspondingly, PSA expression also did not correlate with these pathologic parameters (data not shown).

### Discussion

Prostate-specific membrane antigen is expressed as a prominent Mr 120,000 transmembrane glycoprotein in prostate tissue extracts and seminal plasma.<sup>4-6</sup> It is detected using the mouse MAb 7E11-CS3, produced against a membrane extract of LNCaP cells.<sup>7,16,17</sup> Immunostaining of normal and malignant tissues demonstrated that PSMA expression is highly restricted to prostate tissues<sup>7,12</sup> (also Wright GL Jr, Haley C,

TABLE 5. EXPRESSION OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND PROSTATE-SPECIFIC ANTIGEN IN THE PATIENT'S PRIMARY PROSTATE CARCINOMA AND LYMPH NODE METASTASIS

Patient	Primary carcinoma			Metastatic lymph nodes	
	Gleason <sup>a</sup> sum	Percent cells		Percent cells	
		PSMA	PSA	PSMA	PSA
CA2577	4	0	90	10	25
CA1290	4	5	40	20	70
CA5624	5	70	100	5	5
CA4306	5	10	95	75	100
CA2149	6	5	90	0	95
CA2071	7	15	100	45	95
CA9970	7	40	95	95	95
CA1842	7	40	10	75	25
CA5972	7	85	98	98	93
CA6918	7	20	100	100	90
CA4495	7	95	100	100	100
CA5371	7	25	10	5	30
CA8170	7	45	100	90	50
CA1197	7	00	95	100	100
CA1064	7	60	85	65	100
CA6136	8	65	100	40	100
CA1007	8	90	100	80	95
CA1435	8	70	100	100	90
CA1640	8	10	40	95	50
CA1602	8	55	90	95	80
CA4475	8	75	100	100	100
CA1360	8	5	100	5	75
CA1551	8	85	90	75	95
CA5750	8	80	100	100	90
CA8292	8	75	90	25	25
CA3411	9	30	35	25	80
CA3984	9	70	100	90	100

PSMA = prostate-specific membrane antigen; PSA = prostate-specific antigen.

<sup>a</sup>Gleason sum: 1-4 = well-differentiated; 5-7 = moderately differentiated; 8-10 = poorly differentiated.

TABLE 6. PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND PROSTATE-SPECIFIC ANTIGEN EXPRESSION IN PRIMARY CARCINOMA VERSUS NODE STATUS

Node status	PSMA			PSA		
	No. tested	No. positive	Mean % positive cells*	No. tested	No. positive	Mean % positive cells*
Positive	21	21	54 ± 32.1	21	21	86 ± 26.2
Negative	78	75	49 ± 32.0	78	77	76 ± 27.0

Abbreviations as in Table 5.

\*Mean ± SD.

Beckett ML, unpublished results). Early immunohistochemistry studies showed that MAb 7E11-CS.3 bound the type-2 muscle fibers of normal skeletal muscle; however, a  $^{111}\text{In}$ -labeled immunoconjugate (CYT-356) of MAb 7E11-CS.3 failed to localize to skeletal muscle.<sup>12</sup> Recent studies in our laboratory (Troyer JK, Feng Q, Beckett ML, Wright GL Jr, unpublished results) and at Sloan-Kettering<sup>18</sup> have shown that neither the PSMA glycoprotein nor the PSMA mRNA could be detected in tissue extracts of normal skeletal muscle, suggesting that the observed immunostaining in skeletal muscle is entirely non-specific. Further studies from these laboratories have shown mRNA<sup>18</sup> and PSMA (Troyer et al, unpublished results) in extracts of normal brain, salivary gland, and small intestine by blotting procedures, but not by immunohistochemistry of frozen or formalin-fixed tissue sections<sup>7,12</sup> (also Wright et al, unpublished results). These results suggest either that PSMA expression is below the detection limits of the immunohistochemistry assay or that post-translational modifications mask the PSMA epitope in these tissues. Previous immunohistochemistry studies focused on evaluating the specificity of MAb 7E11-CS.3 tissue reactivity. The present study provides a definitive descriptive immunohistochemistry examination of PSMA expression in normal, benign, and malignant prostate tissues.

Immunoreactivity for PSMA was detected in all types of prostate epithelium, confirming organ specificity rather than prostate carcinoma specificity of this biomarker. Expression of PSMA and PSA in all tissue specimens, with the exception of BPH and bone metastases, paralleled each other. Expression of PSMA in the majority of BPH specimens appeared to be both very heterogeneous and down-regulated. These immunostaining results correlate with the recent observation that PSMA mRNA levels are low to absent in BPH, even though they are high in both normal and malignant prostate tissues.<sup>18</sup> The reason for this phenomenon has yet to be determined. Recent evidence localizing the antigenic epitope recognized by MAb 7E11-CS.3 in the cytoplasmic domain of the PSMA glycoprotein (see Troyer et al, this issue)<sup>19</sup> may indicate that a splicing variant involving the N-terminal amino acid sequence could be responsible for the low PSMA expression in BPH tissues. New antibodies to different PSMA epitopes may assist in addressing this question. Further studies will be required to evaluate this or alternative hypotheses to explain the low PSMA expression in BPH tissues.

The pattern and localization of immunostaining were variable for all prostate tissues examined, with cytoplasmic immunoreactivity observed in all prostate epithelial cells. In contrast to a diffuse cytoplasmic staining, luminal membrane

staining was found in PIN and primary and metastatic carcinoma tissues, with the most prominent membrane staining observed in poorly differentiated primary carcinomas and metastatic tissues. Based on the calculated stain indexes, PSMA was markedly overexpressed in the primary tissues with a high Gleason sum and in both metastatic lymph node and bone lesions. However, in contrast to primary tumors, the metastatic tissues demonstrated less staining heterogeneity. The reason for this apparent up-regulation with more uniform expression in the metastatic tissues is unknown. The effect of hormones on PSMA expression is currently being evaluated.

Although PSMA is an integral transmembrane protein, the cytoplasmic staining observed in prostate epithelium, especially in normal prostate tissues and well-differentiated tumors, could be explained by the location of the epitope in the cytoplasmic domain. Preliminary studies in our laboratory, using both light and electron immunomicroscopy, have demonstrated intracellular as well as membrane staining in cultured LNCaP cells.<sup>20,21</sup> Immunoelectron microscopy showed MAb 7E11-CS.3 localization at the internal region of the plasma membrane, confirming the mapping of the antigenic epitope to the intracellular domain. Besides binding at the internal plasma membrane, MAb 7E11-CS.3 also localized to certain cytoplasmic organelles. Further studies are in progress to determine whether the cellular localization of PSMA observed in LNCaP cells also occurs in prostate tissues. In any event, strong evidence is presented that PSMA is largely expressed intracellularly (ie, intracellular organelles) and at the cytoplasmic face of the plasma membrane of LNCaP cells and prostate tissues.

The observed cytoplasmic staining pattern and localization raise the question of how the  $^{111}\text{In}$ -labeled 7E11-CS.3 immunoconjugate ( $^{111}\text{In}$ -CYT-356) is able to image prostate cancer *in vivo*.<sup>8,9</sup> Epitope-mapping experiments conducted in our laboratory have yet to demonstrate an epitope recognized by MAb 7E11-CS.3 in the extracellular domain of the PSMA glycoprotein (Troyer et al, unpublished results). As stated above, the only epitope recognized by MAb 7E11-CS.3 is located in the cytoplasmic domain. The sequence for this epitope is not found in the extracellular polypeptide region. It is quite possible, however, that the MAb binds to a similar but lower-affinity epitope expressed in the extracellular domain, thereby explaining successful imaging of the prostate cancer. Based on the amino acid sequence, there are numerous glycosylation sites available, suggesting that the extracellular peptide is heavily glycosylated. If this is true, then glycosylation may in fact mask the binding of MAb 7E11-CS.3 to these epitopes. Furthermore, carbohydrates are not part of the

epitope recognized by MAb 7E11-CS.3. Only the linear N-terminal peptide region is required for antibody binding. Based on these observations, the only mechanism for binding of the immunoconjugate to its antigenic target would be by binding to shed antigen in the intercellular spaces or passing through the plasma membrane to reach the epitope. The latter may be possible if the cells are undergoing apoptosis or necrosis. It is entirely possible that the intensely stained focal deposits in the cytoplasm of some malignant cells may represent apoptosis. This possibility is currently being explored. We know that PSMA is shed into prostatic fluid and is present in seminal plasma (Troyer et al, unpublished results), but we have not been able to confirm the initial observations<sup>7</sup> that PSMA is also shed in serum. Further studies will be required to elucidate fully how the <sup>111</sup>In-CYT-356 immunoconjugate images prostate carcinomas.

Expression of PSMA appeared to correlate with tumor grade. When separate stain indexes were calculated for a random cohort of high-grade tumors (SI = 244) containing focal areas of low-grade tumor (SI = 59), the marked overexpression of PSMA in the high-grade areas became more clearly evident. In contrast, 90–100% of all prostate epithelial cells in these tissues intensely expressed PSA. In this study, PSMA expression did not correlate with pathologic stage. However, the majority of tumors examined were stage C disease. Because of the marked overexpression observed in poorly differentiated and metastatic prostate tumors, it might be expected that PSMA expression would correlate with the more aggressive and advanced stage D2 tumors. Additional studies will be required to determine whether this is the case.

Based on our initial observations of the intense membrane expression in the high-grade areas of primary tumors plus the overexpression in the metastatic tumor specimens, we postulated that this pattern of PSMA expression in the primary carcinomas would predict metastasis or tumor progression. However, this was not the case. Evaluation of the nodal status of 99 prostate cancer patients failed to show any correlation with the expression of PSMA in their primary carcinoma; nor was PSMA expression in the primary tumor predictive of extracapsular penetration or seminal vesicle invasion. Because most of the prostate carcinoma specimens were from patients who had had a radical prostatectomy, no attempt could be made in this series to determine the effects of radiation and hormone deprivation therapy on PSMA expression. However, we have noted in preliminary studies that hormone ablation therapy either has no effect on or up-regulates PSMA expression (Grob RM, Haley C, Newhall K, Schellhammer PF, Wright GL Jr, unpublished results). The effect of hormone ablation therapy on PSMA expression will be the subject of a separate report.

This study demonstrates the differential expression of PSMA in normal, benign, and malignant prostate tissues. The unexpectedly low expression in BPH tissues, as compared with normal and malignant prostate tissues, deserves further evaluation to determine the mechanism for the low expression and to exploit this observation as a possible means to differentiate BPH from CaP. We found that PSMA was overexpressed in the poorly differentiated and metastatic tumors. Although PSMA expression in the primary tumor was not predictive of metastatic disease, high expression appears to be

associated with the more aggressive prostate tumor, especially for hormone-refractory cancers. Because of the prominent intracellular location of the antigenic epitope, a mechanism to explain the clinical success of radiologic imaging with CYT-356 remains uncertain. Nevertheless, the restricted specificity, differential prostate tissue expression, and overexpression of PSMA in metastatic tissues support the continued study of this unique prostate tumor-associated biomarker for developing new strategies for the diagnosis and therapy of prostate cancer.

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# PROSTATE-SPECIFIC MEMBRANE ANTIGEN EXPRESSION IS GREATEST IN PROSTATE ADENOCARCINOMA AND LYMPH NODE METASTASES

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## ABSTRACT

**Objectives.** Prostate-specific membrane antigen (PSMA) is an integral membrane protein highly specific for the prostate. PSMA may be clinically useful for predicting outcome in patients with prostate cancer. We compared the expression of PSMA in prostate adenocarcinoma and lymph node metastases in a large series of patients with node-positive cancer.

**Methods.** We studied 232 patients with node-positive adenocarcinoma who underwent bilateral pelvic lymphadenectomy and radical retropubic prostatectomy at the Mayo Clinic between 1987 and 1992. Immunohistochemistry was performed using monoclonal antibody 7E11-5.3 directed against PSMA. For each case, the percentage of immunoreactive cells in benign prostate tissue, adenocarcinoma, and lymph node metastases was estimated in 10% increments. Intensity was recorded using a scale of 0 to 3 (0 = no staining, 3 = highest).

**Results.** Cytoplasmic immunoreactivity for PSMA was observed in all cases in benign epithelium and cancer, and most lymph node metastases. The number of cells stained was lowest in benign epithelium; cancer and lymph node metastases were similar ( $46.2\% \pm 27.5\%$  versus  $79.3\% \pm 18.5\%$  versus  $76.4\% \pm 26.1\%$ , respectively; all pairs  $P < 0.05$ ). Intensity of staining was greatest in primary cancer and lowest in lymph node metastases.

**Conclusions.** PSMA is expressed in benign prostatic epithelium and primary cancer in all cases and in 98% of cases with lymph node metastases. Expression of PSMA was greatest in primary cancer for both percentage and intensity of immunoreactive cells. PSMA expression allows the identification of benign and malignant prostatic epithelium and may be a potentially valuable marker in the treatment of patients with prostate cancer. *UROLOGY* 52: 637-640, 1998. © 1998, Elsevier Science Inc. All rights reserved.

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Prostate cancer has an unknown etiology, variable pathology, and an intricate relationship with endocrine factors. It has the propensity for progression and dedifferentiation, which further adds to the complexity of this disease and limits effective therapies.<sup>1</sup> In 1998 prostate cancer will be diagnosed in an estimated 184,500 men, and 39,200 will die of this cancer.<sup>2</sup> The search for diagnostic and prognostic markers for cancer of the prostate is ongoing.

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Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein with both intracellular and extracellular domains.<sup>3,4</sup> Its function appears to be as a cell-surface peptidase hydrolyzing peptides in prostatic fluid and generating glutamate<sup>4,5</sup> and also acts as a folate hydrolase.<sup>6,7</sup> It is expressed in benign and malignant prostatic epithelium and can be detected immunohistochemically. The antibody used in this study, 7E11-C5.3, recognizes the first six N-terminal amino acids of the cytoplasmic domain. Because PSMA is an integral membrane protein, it is being exploited as a target for antibody-directed imaging and therapeutic targeting modalities.<sup>8-13</sup>

In this report we describe the expression of PSMA in prostatic adenocarcinoma and lymph node metastases in a large series of patients with node-positive cancer.

## MATERIAL AND METHODS

### PATIENTS

We studied select tissue sections from 232 previously untreated patients with prostate adenocarcinoma who underwent bilateral pelvic lymphadenectomy and radical retropubic prostatectomy at the Mayo Clinic between 1987 and 1992. Patients without sufficient cancer tissue available for analysis were excluded. The retrieval of tissue and processing have been previously described.<sup>14,15</sup> In brief, specimens were obtained during radical retropubic prostatectomy and pelvic lymphadenectomy. Each was evaluated by frozen section at the time of surgery and on permanent sections. Each prostate was weighed, measured, and inked. Shave margins were obtained from the apex and base. The remaining prostate was serially sectioned at 4 to 5-mm intervals perpendicular to the long axis of the gland from the apex to the tip of the seminal vesicles. All cases were fixed in neutral buffered formalin overnight and processed routinely to paraffin.

### IMMUNOHISTOCHEMICAL STUDIES

Mouse monoclonal antibody 7E11-5.3 (Cytogen Corp., Princeton, NJ) directed against PSMA was used, as previously described.<sup>15</sup> The immunohistochemical technique included sequential application of diluted primary antibody (PSMA; 20 µg/ml) for 60 minutes, biotinylated goat anti-mouse immunoglobulin (Ig)G and goat anti-rabbit IgG (1:400, Dako Corp., Santa Barbara, Calif) for 30 minutes, and peroxidase-labeled streptavidin (1:500, Dako Corp.) for 30 minutes. Immunoreactivity was visualized by incubation of sections with 3-aminobenzylcarbazole in the presence of hydrogen peroxide. Sections were counterstained with light hematoxylin and mounted with a coverslip. No enzyme pretreatment was used, and microwave antigen retrieval was not necessary (data not shown). Positive and negative controls were run in parallel with each batch and gave appropriate results.

The extent and intensity of staining for this antibody were evaluated in benign prostatic tissue, primary cancer, and lymph node metastases by two of the authors (D.G.B. and A.P.). The percentage of cells exhibiting staining in each case was estimated in 10% increments. Also, a numerical intensity score between 0 and 3 was assigned to each using the following criteria: 0 = no staining; 1 = weak equivocal staining; 2 = unequivocal moderate staining; 3 = strong staining. Only cells showing an intensity of staining greater than 1 were considered positive. In most cases, the staining was unequivocal.

### STATISTICAL STUDIES

Spearman's rank correlations were used to compare the mean percentage of immunoreactive cells for benign epithelium, primary cancer, and lymph node metastases. The significance level was 0.05.

## RESULTS

Cytoplasmic immunoreactivity for PSMA was noted in 100% of cases of benign epithelium and primary cancer and in 98% of lymph node metastases (Fig. 1). Staining was often patchy and heterogeneous. The mean number of cells staining in benign epithelium was 46.2%, which was lower than both cancer and lymph node metastases (79.3% and 76.4%, respectively) (Fig. 2). Each pair, benign versus cancer and cancer versus lymph node metastases, reached statistical significance (0.0016 and <0.0001, respectively). No

staining was observed in urothelium, stroma, or endothelium (Fig. 1).

Adenocarcinoma showed more intense staining than benign epithelium. Lymph node metastases displayed the least intensity of staining (Fig. 3).

### COMMENT

We determined the expression of PSMA in a large series of node-positive prostatic adenocarcinomas and found PSMA in 100% of cases of benign epithelium and primary cancer and 98% of lymph node metastases.

This study confirms other studies of PSMA expression with minor differences (Table I).<sup>1,15-17</sup> Horoszewicz *et al.*,<sup>1</sup> the first to describe monoclonal antibody recognition of PSMA in 1987, identified immunoreactivity in 9 of 9 prostate cancers and 2 of 2 lymph node metastases. Two other studies found PSMA immunoreactivity in the majority of primary cancers and lymph node metastases.<sup>16,17</sup> It is difficult to compare our study with other studies that included patients previously treated with androgen deprivation therapy or radiation.<sup>17</sup> PSMA expression is unchanged or increased after androgen deprivation therapy in primary cancer and lymph node metastases.<sup>18</sup>

PSMA expression is found in normal and malignant nonprostatic tissues.<sup>17,19</sup> One study reported PSMA expression in a subset of proximal renal tubules, duodenal and colonic mucosa, as well as benign and malignant prostate, and lymph node and bone metastases. This study also described intense staining in capillary endothelial cells,<sup>17</sup> although we did not confirm this finding in the current study or our previous report.<sup>15</sup> PSMA expression was also found in brain and salivary gland.<sup>16</sup>

PSMA, as a marker for prostatic epithelium, has many potential therapeutic applications. An immunoconjugate of 7E11-C5.3 termed CYT-356 can be radiolabeled and used to detect lymph node (greater than 5 mm) and bone metastases.<sup>20</sup> CYT-356 labeled with <sup>111</sup>indium (ProstaScint; Cytogen Corp.) predicts extra-prostatic cancer in pelvic lymph nodes.<sup>13</sup> One study described 152 patients with clinically localized cancer who were at high risk for lymph node metastases. Sixty-four patients had histologic evidence of metastases, and 40 of these were detected by the scan (sensitivity of 63%). Twenty-five patients who had positive lymph nodes on scan had lymph nodes free of cancer on histologic evaluation (false positives). At follow-up, 14 of 21 patients developed recurrent cancer.<sup>13</sup> Currently, there are two indications for use of ProstaScint. The first group is patients who are candidates for radical prostatectomy with high probability of metastatic cancer. Second, Prosta-

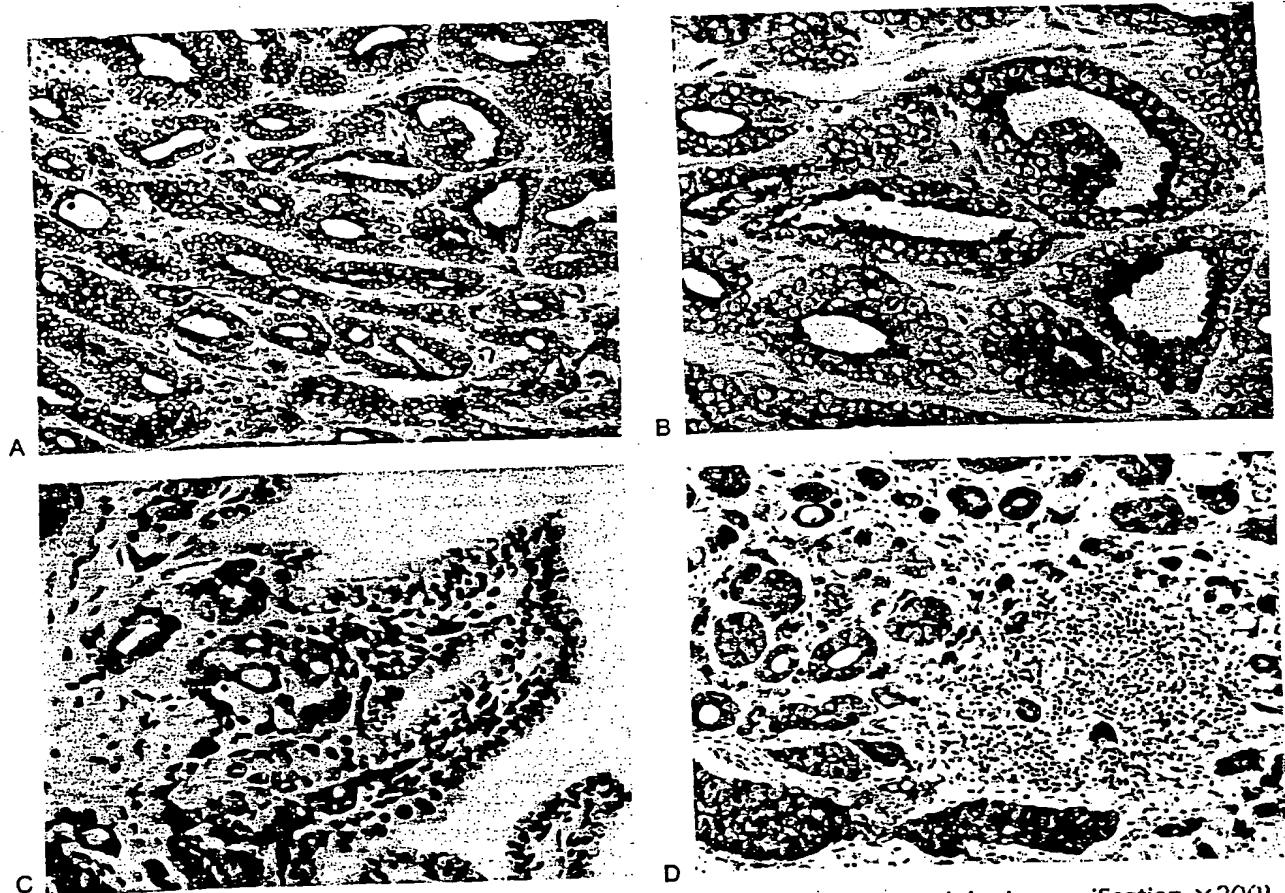


FIGURE 1. Immunohistochemical detection of PSMA. (A) Prostate carcinoma (original magnification  $\times 200$ ). (B) Prostate carcinoma (original magnification  $\times 400$ ). (C) Invasion of prostate cancer into seminal vesicles. Note the absence of PSMA staining in the seminal vesicle epithelium. (D) Lymph node metastases.

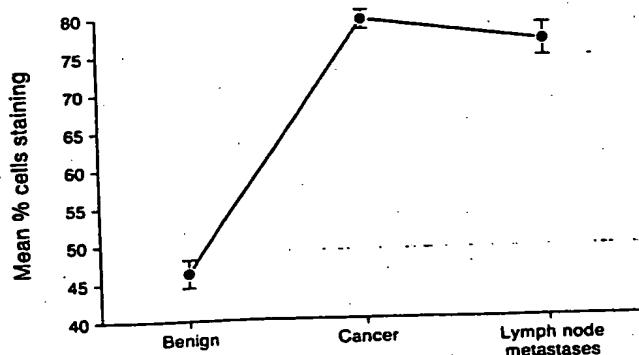


FIGURE 2. Mean percent cells staining for PSMA.

Scint is used to localize foci of prostate cancer suggested by an increasing serum prostate-specific antigen after prostatectomy.<sup>21</sup> Future treatments may include eradicating metastatic deposits by labeling with cytotoxic agents.<sup>5,9</sup>

The PSMA epitope detected by monoclonal antibody 7E11-C5 was initially thought to be intracellular and only accessible in devitalized cells undergoing apoptosis or necrosis.<sup>13</sup> However, a recent study reported that fluorescently labeled 7E11-C5

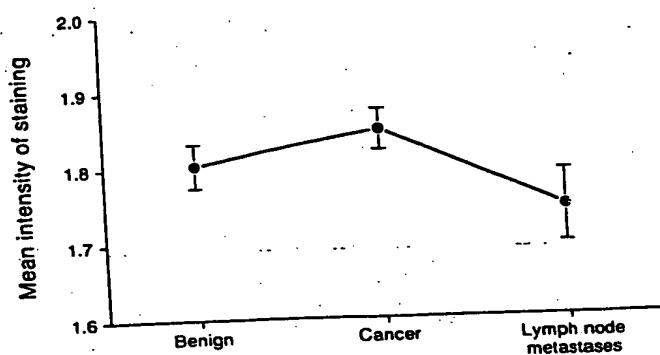


FIGURE 3. Mean intensity of cells staining for PSMA.

specifically binds to PSMA in viable prostate cancer cells,<sup>6</sup> providing laboratory justification for the *in vivo* imaging.

Serum PSMA can be detected using Western blot.<sup>12,22-24</sup> The normal range in healthy individuals between 35 and 55 years of age is 0.14 to 0.22 (relative intensity levels).<sup>12</sup> PSMA serum concentration was higher in patients with disseminated cancer (N+, M+) than in those with localized cancer (T2, T3).<sup>23,24</sup> PSMA concentration may be ele-

TABLE I. PSMA expression in benign and malignant prostate

	Benign Epithelium (%)	PIN (%)	Cancer (%)	Lymph Node Metastases (%)
Horoszewicz <i>et al.</i> <sup>1</sup>	71	—	100	100
Silver <i>et al.</i> <sup>17*</sup>	—	—	94	88
Wright <i>et al.</i> <sup>16</sup>	81	100	95	94
Bostwick <i>et al.</i> <sup>15</sup>	100	100	100	—
Current study	100	—	100	98

KEY: PIN = prostatic intraepithelial neoplasia.

\* Study included patients treated with androgen deprivation or radiation.

vated after treatment, probably reflecting clinical progression or the presence of hormone-resistant cells.<sup>1,12,24</sup> Serum PSMA accurately predicts the stage of prostate cancer or local, regional, or distant metastases in some patients, as shown by the ProstaScint scan.<sup>12</sup>

In summary, we found consistent PSMA immunoreactivity in benign epithelium, primary prostate cancer, and lymph node metastases, with expression highest in cancer and lymph node metastases.

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